ELSEVIER

Molecular and Cellular Probes 18 (2004) 51–57

MOLECULAR AND CELLULAR PROBES

www.elsevier.com/locate/ymcpr

# Twelve hour real-time PCR technique for the sensitive and specific detection of *Salmonella* in raw and ready-to-eat meat products

Jay L.E. Ellingson[a,*], Jennifer L. Anderson[a], Steve A. Carlson[b], Vijay K. Sharma[b]

[a] *Marshfield Clinic Laboratories—Food Safety Services, 1000 North Oak Avenue, Marshfield, WI 54449, USA*

[b] *United States Department of Agriculture—Agricultural Research Service, National Animal Disease Center, Pre-harvest Food Safety and Enteric Diseases Research Unit, Ames, IA 50010, USA*

Received 1 July 2003; accepted for publication 3 September 2003

**Abstract**

Rapid pathogen testing is vital to the food industry. Enzyme immunoassays (EIA) provide reliable negative results in 48 h, but a presumptive positive (suspect) EIA result must be confirmed by traditional culture methods, requiring an additional 72 h. Polymerase chain reaction (PCR) testing technology is accepted as an accurate diagnostic tool. However, traditional PCR techniques can require several days. We sought to develop a rapid, real-time quantitative PCR technique for detecting *Salmonella* spp. in food products. *Salmonella* spp. was inoculated into raw and ready-to-eat beef products. Total DNA was extracted and used as template for PCR amplification in the LightCycler (Roche Diagnostics Corp., Idaho Technology Inc., Idaho Falls, ID) PCR instrument. *Salmonella*-specific PCR primers were designed to amplify a 251 base pair product from the junction of *SipB* and *SipC*. Fluorescently-labeled hybridization probes were designed to anneal to *SipB* and *SipC*. *Salmonella* was detected down to 1 colony forming unit/ml in food products. The results of real-time PCR correlated 100% to those of visual immunoprecipitate and culture. PCR methods using the LightCycler can detect and confirm the presence or absence of *Salmonella* spp. in raw and ready-to-eat beef products within 12 h with increased sensitivity compared to traditional culture and EIA methods.



*Keywords:* Real-time PCR; *Salmonella*; *Sip* Operon; Hybridization probes; Meat products

## 1. Introduction

Food borne illnesses remain a major global health problem. Disease outbreaks resulting from food borne microbial pathogens result in economic losses for the food industry and inflict significant damage to the public health infrastructure. Rapid and accurate pathogen testing is vital in the prevention of such outbreaks.

*Salmonella* infections are the second most common cause of food borne illness in the United States. Approximately 1.4 million cases of salmonellosis are reported in the U.S. each year, more than 500 of which are fatal [1]. The prevention and treatment of this infectious disease has been complicated by the ability of *Salmonella* to acquire resistance to multiple antibiotics [2–8], and the lack of an effective vaccine. In retail meats, 84% of *Salmonella* are resistant to at least one antibiotic and 53% are resistant to at least three antibiotics [9]. These factors have created a need for a rapid, specific, and sensitive detection method for *Salmonella* in contaminated food products.

A combination of enzyme immunoassays (EIA) and culture methods are currently utilized in the testing of food products for detecting *Salmonella* contamination. Using EIA methods, reliable negative sample results can be obtained within 48 h. However, a presumptive positive EIA result must be confirmed by diagnostic culture, which requires an additional 72 h.

Several polymerase chain reaction (PCR) tests to detect *Salmonella* have been developed [2,10–14]. The recent development and availability of rapid real-time thermocyclers have allowed for advancement of traditional PCR techniques. Conventional PCR techniques can require several days and can only provide yes or no answers as to the presence or absence of bacterial pathogens. On the other hand, the testing of samples by real-time PCR with the use

* Corresponding author. Tel.: +1-715-389-3730; fax: +1-715-389-7599.
*E-mail address:* ellingson.jay@marshfieldclinic.org (J.L.E. Ellingson).

0890-8508/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mcp.2003.09.007

of fluorescent hybridization probes can be performed in less than 12 h, and provide both qualitative and quantitative data about the targeted pathogen in food samples. We report the development of a real-time PCR technique for use in the detection of *Salmonella* in raw and ready-to-eat beef products.

## 2. Materials and methods

### 2.1. Sample preparation

Twenty-five gram samples of food products were homogenized in 225 ml of buffered peptone water. For raw meat samples, 4 ml of 0.1% novobiocin was added to the media. Homogenates were incubated for 6 h at 35–37 °C. Samples were removed from the incubator and a 15 ml aliquot of each sample was placed into a 15 ml conical tube. The samples were then returned to the incubator for later testing by visual immunoprecipitate (VIP) and culture methods (see below). The 15 ml aliquots were centrifuged at 2,500 × *g* for 10 min. The supernatant was discarded and the pellets were extracted with the QIAGEN QIAmp DNA mini kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA).

### 2.2. PCR primers and hybridization probes

Oligonucleotides were designed to amplify a 251 base pairs (bp) product spanning from base 2305 to base 2555 of the *sipB–sipC* [2] region of the *Salmonella* genome (GenBank Accession #U25631). The hybridization probes anneal to the upper strand from positions 2464–2497 (upstream) and 2499–2531 (downstream). The sequence and modifications of the primers and probes are presented in Table 1.

### 2.3. PCR amplification

Three microliters of extracted DNA was used as template for PCR amplification in the LightCycler PCR instrument (U.S. patent 6,174,670; Roche Diagnostics Corp., Idaho Technology Inc., Idaho Falls, ID). The amplification mixture (20 μl final volume) contained the following concentrations of reactants: 1 μM of each primer, 0.2 μM upstream probe, 0.4 μM downstream probe, and 3.5 mM MgCl. DNA master was added according to manufacturer's directions (Roche LightCycler-DNA master hybridization probes kit). Each set of samples included a negative control (in which deionized, distilled water was substituted for sample). Master mix and extracted DNA were placed into a capillary tube, sealed, centrifuged, (2000 × *g* for 1 min) and placed into the LightCycler carousel.

DNA was amplified as follows: 2 min at 95 °C, 45 heating/cooling cycles (each cycle being 0 s at 95 °C, 5 s at 53 °C with a single acquisition during each cycle, and 10 s at 72 °C). DNA was denatured from hybridization probes as follows: 0 s at 95 °C, 15 s at 45 °C, and 0 s at 95 °C with a 0.1 °C/s slope and continuous acquisition. Cooling cycle was 30 s at 40 °C.

### 2.4. Sensitivity assays

Sensitivity assays were designed to determine the limit of detection in and out of food products. Before each

Table 1
PCR primers and hybridization probes

| |
|---|
| *PCR primers* |
| Forward: 5′-ACAGCAAAATGCGGATGCTT-3′ |
| Reverse: 5′-GCGCGCTCAGTGTAGGACTC-3′ |
| *Hybridization probes* |
| Upstream: 5′-GCAATCCGTTAGCGCTAAAGATATTCTGAATAGT-Fluorescein-3′ |
| Downstream: 5′-LC RED 640-650TTGGTATTAGCAGCAGTAAAG-TCAGTGACCTGG-Phos-3′ |

GenBank Accession #U25631.

Table 2
Comparison of 12 h PCR to VIP and culture methods in cross-reactivity tests

| Sample ID | PCR assay | VIP assay | Culture (if VIP positive) |
|---|---|---|---|
| *Bacillus cereus* | negative | negative | n/a |
| *Citrobacter freundii* | negative | negative | n/a |
| *Enterococcus faecalis* | negative | negative | n/a |
| *Escherichia coli* | negative | negative | n/a |
| *Escherichia coli* O157:H7 | negative | negative | n/a |
| *Klebsiella pneumoniae* | negative | negative | n/a |
| *Listeria innocua* | negative | negative | n/a |
| *Listeria monocytogenes* | negative | negative | n/a |
| *Pseudomonas aeruginosa* | negative | negative | n/a |
| *Salmonella abaetatuba* | positive | positive | positive |
| *Salmonella agona* | positive | positive | positive |
| *Salmonella cholerasuis* | positive | positive | positive |
| *Salmonella derby* | positive | positive | positive |
| *Salmonella dublin* | positive | positive | positive |
| *Salmonella enterica* serotype Typhimurium | positive | positive | positive |
| *Salmonella enterica* serotype Typhimurium DT104 | positive | positive | positive |
| *Salmonella enteritidis* | positive | positive | positive |
| *Salmonella heidelberg* | positive | positive | positive |
| *Salmonella litchfield* | positive | positive | positive |
| *Salmonella monterideo* | positive | positive | positive |
| *Salmonella mueuchen* | positive | positive | positive |
| *Salmonella newport* | positive | positive | positive |
| *Salmonella ohio* | positive | positive | positive |
| *Shigella flexneri* | negative | negative | n/a |
| *Shigella sonnei* | negative | negative | n/a |
| *Staphylococcus aureus*(coagulation +) | negative | negative | n/a |

VIP, visual immunoprecipitate.

experiment, Petroff-Hausser counts were done on a log phase culture of *Salmonella enterica* ser. Abaetatuba. The following concentrations of *Salmonella abaetatuba* were added to 25 g of several different food products in 225 ml buffered peptone water: 1 colony forming unit (CFU)/ml, 10, and 100 CFU/ml. The food products were mechanically homogenized and aliquots were plated in duplicate on xylose lysine desocholate agar. These plates and the homogenized food products were placed into a 35–37 °C incubator. After 6 h, the food products were concentrated and the DNA was extracted using the method described earlier and used as template in real-time PCR experiments. The CFUs on desocholate plates were enumerated after 24 h.

### 2.5. Control Salmonella strains

Fifteen strains of *Salmonella* were obtained from the United States Department of Agriculture. Other common foodborne bacteria were tested for cross reactivity to the hybridization probes (Table 2). All bacterial strains were

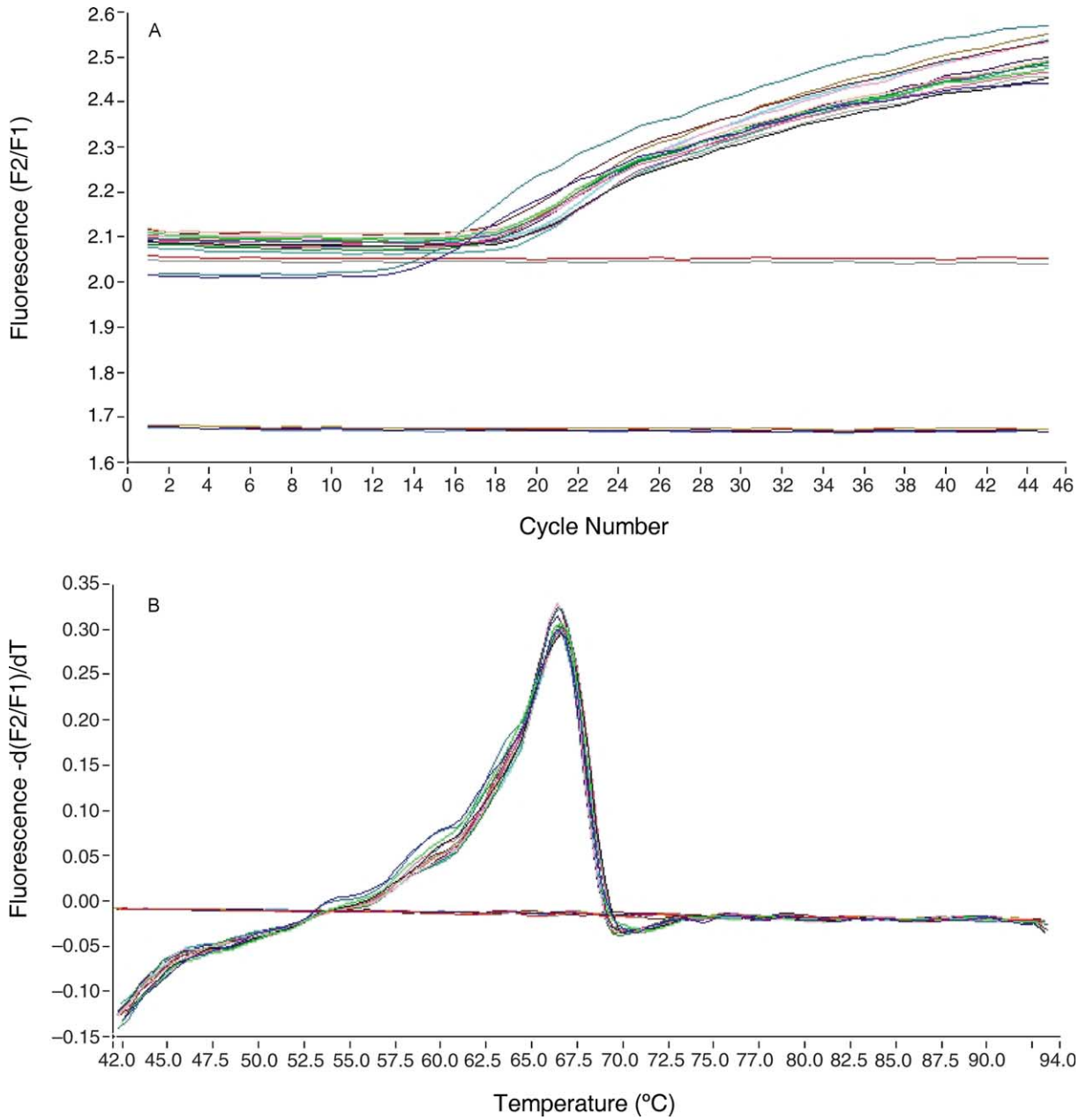


Fig. 1. Amplification and melting curves of 10 strains of *Salmonella* sp. (*S. abaetatuba*, *S. agona*, *S. dublin*, *S. enteritidis*, *S. hadar*, *S. heidelberg*, *S. newport*, *S. seftenberg*, *S. enterica* serotype Typhimurium, and *S. enterica* serotype Typhimurium phasetype DT104). Identical melting curves were exhibited by 15/15 *Salmonella* strains, demonstrating the specificity of the hybridization probes for the *SipB/C* region sequence. Only the negative control exhibited no amplification or melting curve. (A) Superimposed amplification curves of duplicate samples. (B) Superimposed melting curves for duplicate samples.

grown up in brain-heart infusion both and extracted using the method described above.

### 2.6. EIA and culture techniques

All samples were tested in accordance with the instructions for VIP (AOAC Official Method: 999.09). Samples that tested positive by the VIP method were confirmed by traditional culture methods as described in the Federal Drug Administration Bacteriological Analytical Manual, 8th edition.

## 3. Results

### 3.1. Assay specificity

LightCycler hybridization probe PCR detected 15 of 15 different standard strains of *Salmonella*. Ten duplicate assessments are shown in Fig. 1. The hybridization probes did not anneal with any of the other bacterial species tested, including *Bacillus cereus*, *Citrobacter freundii*, *Enterococcus faecalis*, *Escherichia coli*, *Escherichia coli* O157:H7, *Klebsiella pneumoniae*, *Listeria innocua*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Shigella sonnei* and *Staphylococcus aureus* (coagulase +) (Table 2).

Table 3
Comparison of 12 h PCR to VIP and culture methods for detection of *Salmonella* spp. in contaminated specimens and food products

| Sample ID | PCR assay | VIP assay | Culture (if VIP positive) |
|---|---|---|---|
| Human | positive | positive | positive |
| 1 | positive | positive | positive |
| 2 | positive | positive | positive |
| 3 | positive | positive | positive |
| 4 | positive | positive | positive |
| 5 | positive | positive | positive |
| 6 | positive | positive | positive |
| 7 | positive | positive | positive |
| Bovine | | | |
| 129 | positive | positive | positive |
| 130 | positive | positive | positive |
| 131 | positive | positive | positive |
| 132 | positive | positive | positive |
| 133 | positive | positive | positive |
| 134 | positive | positive | positive |
| 135 | positive | positive | positive |
| 136 | positive | positive | positive |
| 137 | positive | positive | positive |
| Food | | | |
| 16158 [serogroup C2 and C3(O):210(H)] | positive | positive | positive |
| 22312 | positive | positive | positive |
| 30977 | positive | positive | positive |
| 33982 | positive | positive | positive |
| 60738 [serogroup B(O):r(H)] | positive | positive | positive |
| 65552 [serogroup E1(O):poly A–Z(H)] | positive | positive | positive |
| 65572 [serogroup E1(O):poly A–Z(H)] | positive | positive | positive |
| 68528 [serogroup B(O):r(H)] | positive | positive | positive |
| 71541 [serogroup C1(O):poly A–Z(H)] | positive | positive | positive |
| 94844 [serogroup B(O):r(H)] | positive | positive | positive |

VIP, visual immunoprecipitate.

Table 4
Sensitivity tests using DNA extracted from cultured *Salmonella* Ser. Abaetatuba

| Plate ID | Petroff–Hausser count | Plate 1 CFUs/ 100 μl | Plate 2 CFUs/ 100 μl | Average | Genomic equivalents/ reaction |
|---|---|---|---|---|---|
| sal1 | 200 organisms | 196 | 178 | 187 | 6 |
| sal2 | 1000 organisms | 778 | 703 | 740 | 23 |
| sal3 | 1500 organisms | 1076 | 1180 | 1132 | 34 |
| sal4 | 2000 organisms | 2468 | 1700 | 2084 | 63 |
| sal5 | 3000 organisms | 3472 | 3388 | 3430 | 103 |

CFU, colony forming units.

### 3.2. Suspected contaminated isolates

Twenty-six isolates presumptively identified as with *Salmonella* from humans, cattle, and food products were tested to further validate the utility of real-time PCR for positive identification of suspected isolates of *Salmonella*. These isolates were obtained from Marshfield Clinic/Saint Joseph's Hospital Joint Venture Laboratory, Marshfield Clinic Laboratories Veterinary Laboratory, and Marshfield Clinic Laboratories—Food Safety Services. Of these 26 isolates, 100% tested positive for *Salmonella* by real-time PCR, VIP, and culture methods (Table 3).

### 3.3. Sensitivity tests

In previous tests, amplification and analysis revealed that approximately six genomic equivalents of *Salmonella enterica* ser. *abaetatuba* were within the range of detection (Table 4, Fig. 2). Later experiments in spiked raw and ready-to-eat beef products showed that 1 organism/ml of *Salmonella* Abaetatuba can be detected after a 6 h incubation and concentration step (Table 5, Fig. 3).

## 4. Discussion

The real-time PCR method described in this report is ideal for rapid detection of *Salmonella* sp. in raw and ready-to-eat meat products. This method dramatically reduces the reporting time required for reporting detection results as compared to standard EIA, diagnostic culture, and other PCR methods. The fluorogenic probes utilized in the real-time PCR proved to be species specific for *Salmonella* and resulted in detection specificity that correlated 100% to

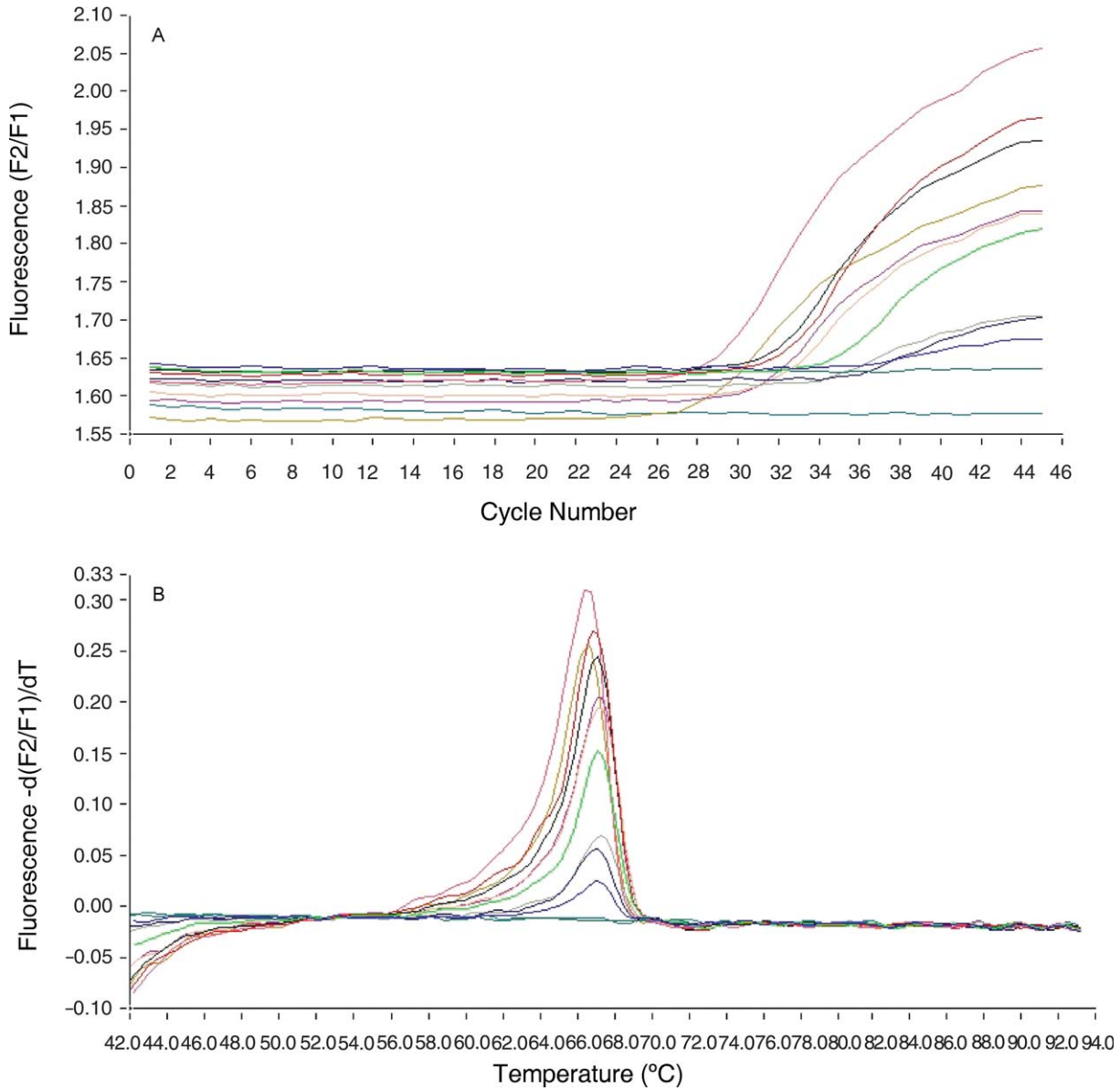


Fig. 2. Amplification and melting curves showing the detection limits of LightCycler PCR for DNA prepared from cultured *Salmonella* ser. Abaetatuba. (A) Superimposed amplification curves of duplicate samples (Table 4). (B) Superimposed melting curves for duplicate samples (Table 4).

those of EIA and culture methods (Tables 2 and 3). Results for real-time PCR were obtained in 12 h as compared to 2–5 days for EIA and culture methods. The method described also has the added advantage of being run on the LightCycler. This instrument allows for reactions to be monitored while amplification is taking place, and is equipped with software that can quantify the number of genomic equivalents in each reaction. The use of real-time quantitative PCR with hybridization probes provided fast and accurate detection of *Salmonella* species in food

Table 5
Sensitivity in ready-to-eat (RTE) and raw (R) beef products spiked with *Salmonella* ser. Abaetatuba after incubation and concentration

| Plate ID | Petroff–Hausser count | Concentration of *Salmonella* (250 ml) | Plate 1 (CFUs/0.5 ml) | Plate 2 (CFUs/0.5 ml) | Organisms/ml |
|---|---|---|---|---|---|
| RTE250 | 250 organisms | 1 organism/ml | 0 | 1 | 1 |
| RTE2500 | 2500 organisms | 10 organisms/ml | 7 | 5 | 12 |
| RTE25000 | 25,000 organisms | 100 organisms/ml | 42 | 67 | 109 |
| R250 | 250 organisms | 1 organism/ml | 1 | 1 | 2 |
| R2500 | 2500 organisms | 10 organisms/ml | 3 | 10 | 13 |
| R25000 | 25,000 organisms | 100 organisms/ml | 56 | 38 | 94 |

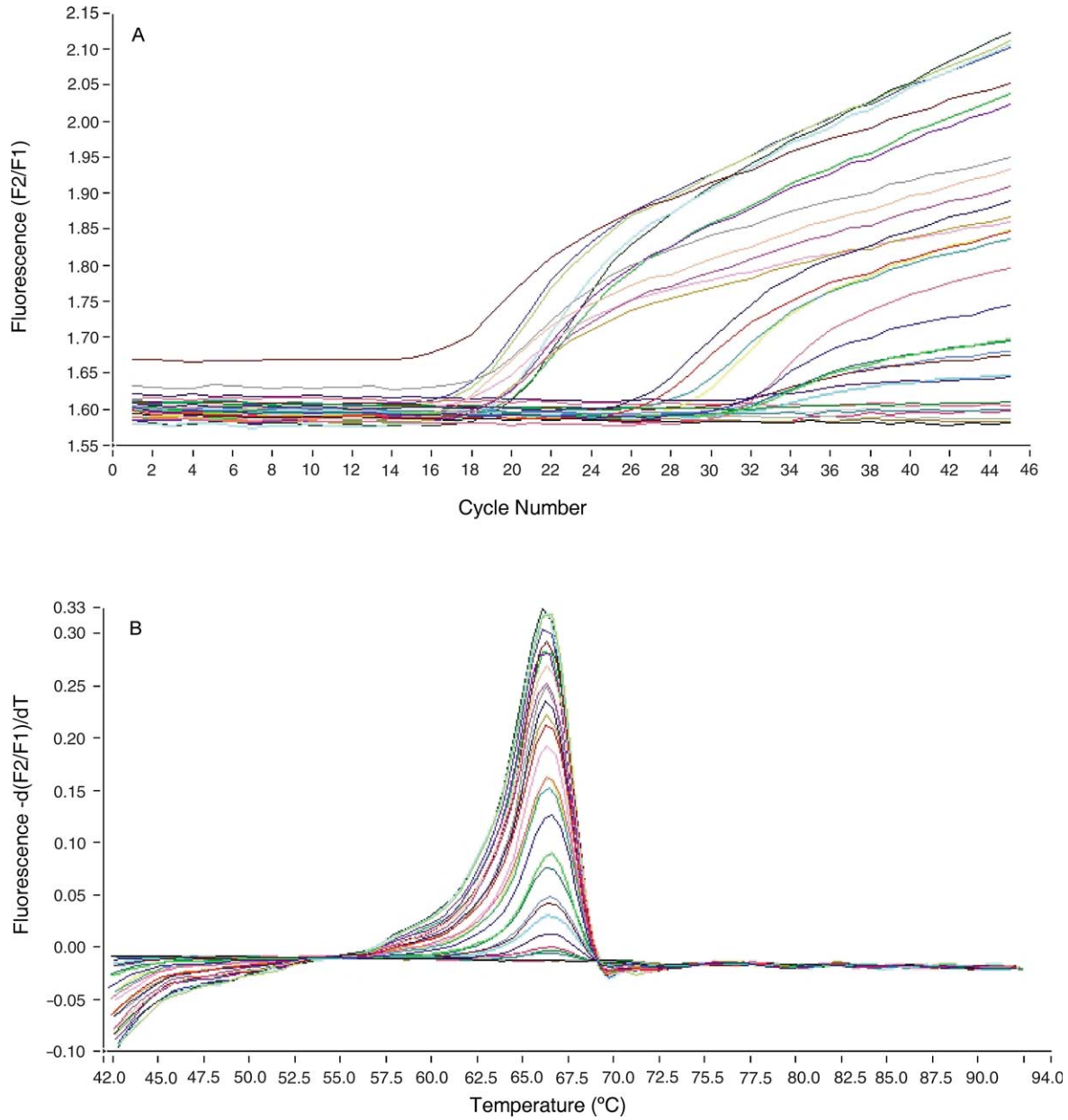


Fig. 3. Amplification and melting curves showing the detection limits of LightCycler PCR in raw and ready-to-eat beef products spiked with *Salmonella* ser. Abaetatuba. (A) Superimposed amplification curves of duplicate samples (Table 5). (B) Superimposed melting curves for duplicate samples (Table 5).

products with at least equal sensitivity and specificity as currently used methods.

We believe that our method of PCR is more sensitive because it requires fewer organisms for detection than EIA or culture methods. It has been shown that EIA methods require the presence of at least $10^3$ organisms in order to be able to detect *Salmonella* [15–17]. The PCR method described in this report requires the presence of between 1–10 organisms for detection. Because this method requires the presence of fewer organisms for detection, the enrichment phase of our assay is considerably shorter.

The use of two fluorescently-labeled oligonucleotide hybridization probes within the desired region of amplification provided specific detection of *Salmonella*. Because the hybridization probes are sequence-specific, they are designed to anneal only to the target DNA sequence. During the annealing step of PCR, the probes hybridize to the target DNA sequence. Then the fluorochrome on the 3′ end of the first probe is excited by an external light source. The close proximity (1 bp) of this first fluorochrome to a second fluorochrome on the 5′ end of a second probe allows for the transfer of energy to take place. The second probe then emits a measurable amount of light. Because this measurable amount of light is proportional to the amount of product being made, reaction progress can be monitored during each run.

The use of hybridization probes in combination with subsequent melting curve data aids in product confirmation. A melting step can be performed at the end of amplification. During this step the chamber heats slowly while continuously obtaining fluorescence readings from each sample. A drop in fluorescence will be detected as the lower melting probe denatures from the amplified product. The fluorescence values are then plotted against temperature to obtain the melting curve.

The ability to invade host cells is a key pathogenic factor of *Salmonella*. The (*Salmonella* invasion proteins) *SipB/C* region of the *Salmonella* genome has been previously shown to encode for proteins that control the invasion of *Salmonella* into host. The *SipB/C*-encoded proteins in conjunction with other virulence factors enable *Salmonella* to invade and damage host cells [18,19]. It was hypothesized that these primer and probe sets would only detect invasive strains of *Salmonella*. By focusing in on this group of strains we eliminate possible positives from *Salmonella* that do not have the ability to invade cells and cause disease [20].

## 5. Conclusions

As described in this paper, quantitative PCR methods using the LightCycler can both detect and confirm the presence or absence of *Salmonella* species within 12 h upon receipt of sample, with increased sensitivity compared to the currently used diagnostic culture and EIA methods. The ability to receive *Salmonella* test results in a more timely fashion will prevent recalls of contaminated products by stopping the contaminated products from being introduced into the marketplace. This will not only prevent many people from becoming infected with *Salmonella*, it will also benefit food manufacturing companies by extending their product's shelf-life by several days and saving them the cost of warehousing their food products while awaiting pathogen testing results.

## Acknowledgements

Thanks to Marshfield Clinic Research Foundation for providing assistance in the preparation of this manuscript through the services of Graig Eldred, PhD and Alice Stargardt, and Dirk Vevea for laboratory assistance.

## References

[1] Centers for Disease Control, Division of Bacterial and Mycotic Diseases. Salmonellosis. http://www.cdc.gov/ncidod/dbmd/diseaseinfo/salmonellosis_t.htm. Accessed March 29, 2002

[2] Carlson SA, Bolton LF, Briggs CE, et al. Detection of multiresistant *Salmonella Typhimurium* DT104 using multiplex and fluorgenic PCR. Mol Cell Probes 1999;13:213–22. doi: 10.1006/mcpr.1999.0240.

[3] Dravid M, Bhore AV, Joshi BN, Phadke S. Salmonella newport isolations from a general hospital in Pune. J Commun Dis 1989;21:133–6.

[4] Dunne EF, Fey PD, Kludt P, et al. Emergence of domestically acquired ceftriaxone-resistant *Salmonella* infections associated with AmpC beta-lactamase. J Am Med Assoc 2000;284:3151–6.

[5] Glynn MK, Bopp C, Dewitt W, Dabney P, Mokhtar M, Angulo FJ. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. N Engl J Med 1998;338:1333–8.

[6] Karmaker S, Biswas D, Shaikh NM, Chatterjee SK, Kataria VK, Kumar R. Role of a large plasmid of *Salmonella* Typhi encoding multiple drug resistance. J Med Microbiol 1991;34:149–51.

[7] Shanahan PM, Karamat KA, Thomson CJ, Amyes SG. Characterization of multi-drug resistant *Salmonella* Typhi isolated from Pakistan. Epidemiol Infect 2000;12:9–16.

[8] Matsumoto M, Suzuki M, Hiramatsu R, et al. An increase in multidrug resistant isolates of *Salmonella Typhimurium* from healthy carriers in Aichi, Japan. Jpn J Infect Dis 2000;53:164–5.

[9] White DG, Zhao S, Sudler R, et al. The isolation of antibiotic-resistant salmonella from retail ground meats. N Engl J Med 2001;345:1147–54.

[10] Hirose K, Itoh K, Nakajima H, et al. Selective amplification of *tyv* (rfbE), *prt* (rfbS), *viaB*, and *fliC* genes by multiplex PCR for identification of *Salmonella enterica* serovars Typhi and Paratyphi A. J Clin Microbiol 2002;40:633–6. doi: 10.1128/JCM.40.02.633-636.2002.

[11] Hsih HY, Tsen HY. Combination of immunomagnetic separation and polymerase chain reaction for the simultaneous detection of *Listeria* monocytogenes and *Salmonella* spp. in food samples. J Food Prot 2001;64:1744–50.

[12] Knutsson R, Lofstrom C, Grage H, Hoorfar J, Radstrom P. Modeling of 5′ nuclease real-time responses for optimization of a high-throughput enrichment PCR procedure for *Salmonella enterica*. J Clin Microbiol 2002;40:52–60. doi: 10.1128/JCM.40.1.52-60.2002.

[13] Lampel KA, Orlandi PA. Polymerase chain reaction detection of invasive *Shigella* and *Salmonella enterica* in food. Meth Mol Biol 2002;179:235–44.

[14] Eyigor A, Carli KT, Unal CB. Implementation of real-time PCR to tetrathionate broth enrichment step of Salmonella detection in poultry. Lett Appl Microbiol 2002;34:37–41. doi: 10.1046/j.1472-765x.2002.01036.x.

[15] Singlepath® *Salmonella*. GLISA-rapid test (Gold Labelled Immuno-Sorbent Assay) for the qualitative detection of Salmonella spp. in food. http://www.merck.de/english/services/chemdat/catalogs/mibio/tedisdata/prods/4980-1_04140_0001.html. Accessed May 1, 2003

[16] Feldsine PT, Mui LA, Forgey RL, Kerr DE. Equivalence of visual immunoprecipitate assay (VIP®) for *Salmonella* for the detection of motile and nonmotile *Salmonella* in all foods to AOAC culture method: collaborative study. J AOAC Int 2000;83:888–902.

[17] Stewart DS, Reineke KF, Tortorello ML. Comparison of assurance gold *Salmonella* EIA, BAX for screening/*Salmonella*, and GENE-TRAK *Salmonella* DLP rapid assays for detection of *Salmonella* in alfalfa sprouts and sprout irrigation water. J AOAC Int 2002;85:395–403.

[18] Kaniga K, Tucker S, Trollinger D, Galan JE. Homologs of the *Shigella* IpaB and IpaC invasins are required for *Salmonella* Typhimurium entry into cultured epithelial cells. J Bacteriol 1995;177:3965–71.

[19] Zhao L, Ezak T, Kawamura Y, Hirose K, Watanabe H. Vi-Suppressed wild strain *Salmonella* Typhi cultured in high osmolarity is hyperinvasive toward epithelial cells and destructive of Peyer's patches. Microbiol Immunol 2001;45:149–58.

[20] Ginocchio CC, Rahn K, Clarke RC, Galan JE. Naturally occurring deletions in the centisome 63 pathogenicity island of environmental isolates of *Salmonella* spp. Infect Immun 1997;65:1267–72.